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# Selection of mutant CHO clones resistant to murine gammaherpesvirus 68 infection

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**Abstract**

Murine gammaherpesvirus 68 (MHV68) is used as a model to study gammaherpesvirus pathogenesis both in tissue culture systems and *in vivo*. We used a gene-trapping approach to get insight into cellular factors involved in MHV68 infection. By generating a library of gene-trapped CHO cells, we were able to isolate several clones that exhibited various degrees of resistance to MHV68-induced cytopathic effect. Clones that showed the highest degree of resistance were affected at the early stage of the viral cycle, with the vast majority of these clones being deficient for heparan sulfate (HS) expression at the cell surface. Heparan sulfate expression could be restored in all the HS-deficient clones by expression of EXT1, an enzyme that is essential for the biosynthesis of HS. Consistent with the role of HS in viral entry, HS-deficient CHO cells did not support viral internalization. Cell surface heparan sulfate proteoglycans (HSPG) are mostly composed of HS chains attached to two families of core proteins, the transmembrane syndecans and the GPI-anchored glypicans. Treatment of CHO cells with phosphatidylinositol-specific phospholipase C (PI-PLC) did not significantly affect the level of HS expression, indicating that the glypicans are not a major source of HSPG in CHO cells. By contrast, treatment of CHO cells with PMA, a drug known to accelerate syndecan shedding, resulted in a decrease in both HS expression and susceptibility to MHV68; these effects were abolished by TIMP-3, a specific inhibitor of syndecan shedding. All together, our results confirm the essential role of HS in MHV68 infection and identify the syndecans as a major source of HSPG used by the virus as coreceptors to infect CHO cells.


*Keywords:* Gammaherpesvirus; MHV68; Heparan sulfate; Syndecan

## Introduction

Gammaherpesviruses are characterized by their ability to establish latent infections in B cells. Two human gammaherpesviruses, Epstein–Barr virus (EBV) and Kaposi's Sarcoma associated Herpesvirus (KSHV), are important human pathogens that are associated with both lymphoid and non-lymphoid tumors (Damania, 2004). First discovered in association with endemic Burkitt's lymphoma (Epstein et al., 1964), EBV is associated with several other B cell malignancies (such as post-transplant lymphoproliferative diseases, and a subset of Hodgkin's lymphomas) and with epithelial cancers (such as nasopharyngeal carcinoma and a subset of gastric carcinomas) (Pagano, 1999; Young and Rickinson, 2004). KSHV is the etiologic agent of Kaposi's sarcoma (Chang et al., 1994), a skin cancer prevalent in AIDS patients, as well as two AIDS-associated lymphoproliferative disorders, primary effusion lymphoma (Birkmann et al.) and Castelman disease (Dourmishev et al., 2003). With the advent of highly active anti-retroviral therapy (HAART) regimen, the frequency of these diseases has decreased in developed countries. However, they remain high in other regions of the globe particularly in several areas of Africa where the prevalence of HIV is high. EBV and KSHV infect only humans and do not replicate efficiently in cell lines (Ahearn et al., 1988; Bechtel et al., 2003; Blackbourn et al., 2000; Knox et al., 1996; Renne et al., 1998). Our understanding of gammaherpesvirus pathogenesis has been severely hindered by the absence of animal models as well as the lack of good tissue culture systems. Murine gammaherpesvirus 68 (MHV68) is a natural pathogen of rodents (Blaskovic et al., 1980). Viral genome structure and sequence analysis indicate that MHV68 is closely related to KSHV and EBV. MHV68 genome encodes approximately 80 open reading frames, at least 63 of which are

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collinear and homologous to KSHV (Virgin et al., 1997). Like EBV and KSHV in humans, MHV68 can induce lymphoproliferative disease and high-grade lymphomas in long-term infected mice (Tarakanova et al., 2005). After intranasal inoculation, MHV68 infects epithelial cells in the lungs and spreads to the lymphoid tissue where it infects B cells, dendritic cells and macrophages (Flano et al., 2000; Stewart et al., 1998; Sunil-Chandra et al., 1992). The virus establishes a life-long infection, mostly in B cells. MHV68 replicates efficiently in several fibroblast and epithelial cell lines, greatly facilitating the study of host–virus interactions at the cellular level. For all these reasons, MHV68 represents an invaluable tool to understand the interactions between gammaherpesviruses and their hosts, both *in vitro* and *in vivo* (Simas and Efstathiou, 1998).

Viruses hijack the whole cell machinery to suit their needs. Forward genetics has proved useful in numerous examples to identify cellular cofactors used by pathogens to infect host cells. In particular, various viral receptors and coreceptors were identified by using cDNA transfer into non-permissive cell lines (Albritton et al., 1989; Chan et al., 2001; Feng et al., 1996; Mendelsohn et al., 1989; Montgomery et al., 1996; Warner et al., 1998). However, the major limitation of such approach is the limited number of naturally non-permissive cells, and in particular cells that are affected at a single step of the viral cycle. Generation of loss-of-function mutants by random mutagenesis in mammalian cell lines is still a major challenge. Indeed, diploidy creates a major obstacle because both copies of a gene must be altered. Yet numerous studies reported the selection of lack-of-function mutants of the Chinese Hamster Ovary (CHO) cell line (Carabeo and Hackstadt, 2001; Chang et al., 1993; Esko et al., 1986; Higaki et al., 2001; Hubbard et al., 1994; Liu and Leppla, 2003; Mento and Siminovitch, 1981; Metherall et al., 1991; Moehring et al., 1993; Moehring and Moehring, 1977; Nobukuni et al., 2005; Watson et al., 1991). CHO cell mutants have been obtained at high frequencies, mostly using chemical mutagenesis and to a lesser extent insertional mutagenesis, indicating that these cells have substantial functional hemizygosity at many loci. Gene trapping is a powerful approach for forward genetics. Random mutagenesis by insertion of a gene trap vector was originally developed in embryonic stem (ES) cells in order to perform genome-wide functional genomics in mice (Raymond and Soriano, 2006; Stanford et al., 2001). Gene trap vectors contain a reporter gene that is expressed only upon insertion within a gene thus allowing selection of cells in which a copy of a gene has been disrupted. Furthermore, the altered gene is physically marked by the vector, thus facilitating its identification.

We decided to use a gene-trapping approach to identify cellular factors important for MHV68 infection. The hypodiploid CHO cell line is highly susceptible to MHV68 infection. We generated a library of gene-trapped CHO cells and selected for cells surviving MHV68 infection. We were able to isolate about 100 clones, most of them showing only partial resistance to MHV68-induced cytopathic effect. We focused on clones affected in the early stages of the viral cycle and found that the majority of them were deficient for heparan sulfate expression at the cell surface, due to a defect in EXT1 expression. These clones were the most resistant to MHV68 infection thus strengthening the essential role of HS in viral infection. Heparan sulfate is critically required for the binding of several viruses and bacterias. The present results reveal that forward genetic screens involving the selection of mutant CHO cells resistant to any of these microorganisms will likely be biased toward the selection of HS-deficient cells. Our findings suggest that one way to avoid this setback might be to use CHO cells stably expressing EXT1. Finally, to further characterize MHV68-HS interaction, we determined the nature of the heparan sulfate proteoglycan (HSPG) in CHO cells. We provide evidence that MHV68 uses HS carried by syndecan molecules to infect CHO cells.

## Results

### *Characterization of MHV68 infection in CHO cells*

We first sought to verify if the hypodiploid CHO cell line was suitable for a forward genetic screen aimed at identifying cellular factors involved in MHV68 infection. To determine the degree of susceptibility to MHV68, CHO cells were infected with various multiplicity of infection (MOI) using a virus that expresses GFP upon entry of the viral DNA into the nucleus. The percentage of GFP-positive cells was determined by flow cytometry 24 h post infection. As shown in Fig. 1A, CHO cells are highly permissive to viral infection, with more than 80% of infected cells using an MOI as low as 0.25. In all our experiments, the MOI is estimated according to the viral titer measured by a standard plaque assay on NIH3T3 cells. Using the same flow cytometry assay as above, we found that CHO are remarkably more susceptible than BHK21 and NIH3T3 cell lines, two cell types that are routinely used for MHV68 production and titration respectively (data not shown). Infection led to a complete cytopathic effect within 48 to 72 h, manifested by a rounding of cells and detachment from the plastic surface. To evaluate virion production in CHO cells, CHO and BHK21 cells were infected with MHV68-GFP, washed to remove the input virus, and a sample of the culture supernatant was taken either at 3 h or 48 h after infection. These samples were then used to infect BHK21 cells and the extent of infection was determined by flow cytometry after 24 h. As shown in Fig. 1B, supernatant from BHK21-infected cells led to a high efficiency of infection by contrast to supernatant from CHO cells. These results indicate that, whereas CHO cells are highly permissive to MHV68 infection and show a complete cytopathic effect, virion production is restricted in these cells. The presence of a massive cytopathic effect will greatly ease the isolation of resistant clones. Moreover, the limited viral production will potentially allow the selection of partially resistant clones by lowering the stringency of the screen. Isolation of such clones is important. For example, herpesviruses can use different surface receptors (Akula et al., 2002; Kaleeba and Berger, 2006; Rappocciolo et al., 2006; Spear, 2004). The presence of more than 1 receptor at the cell surface would render the isolation of receptor-deficient cells impossible in a stringent screen with continuous viral production.

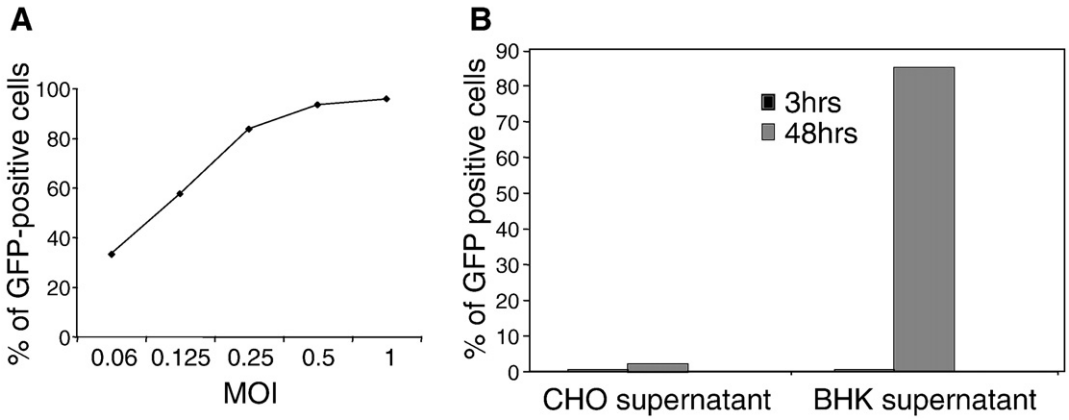


Fig. 1. Characterization of MHV68 infection in CHO cells. (A) CHO cells were infected with MHV68-GFP at the indicated MOI and the percentage of GFP-expressing cells was determined by flow cytometry 24 h post-infection. (B) BHK21 or CHO cells were infected with MHV68-GFP at an MOI of 0.1. Cells were rinsed after 2 h to remove the viral inoculum, and incubated with growth media. Samples of the growth media were then collected at 3 h or 48 h post infection. The presence of viral particles in the growth media was determined by infecting BHK21 cells with undiluted supernatant. The percentage of GFP-positive cells was determined by using flow cytometry 24 h later.

*Generation of a gene-trapped CHO library and selection of MHV68-resistant clones*

The ROSA gene trap vectors developed by the Soriano laboratory (Chen and Soriano, 2003; Friedrich and Soriano, 1991) have been successfully used in numerous studies to create mutations into the genome of mouse ES cells (Komada et al., 2000; Komada and Soriano, 2002). The ROSA vectors include a splice acceptor site 5′ to a promoterless reporter gene encoding a fusion protein of βgalactosidase and neomycin phosphotransferase (βgeo). Expression of the reporter gene requires transcription from a cellular promoter. Because of the presence of a splice acceptor site, insertion of the vector in an intron leads to expression of a fusion mRNA and consequently a mutation in the cellular gene. Moreover, this leads to expression of the βgeo protein, conferring neomycin (G418) resistance.

We performed a small-scale experiment to determine the trapping efficiency in CHO cells using the ROSAβgeo * vector (Komada et al., 2000). Wild-type CHO cells are poorly permissive to transduction with retroviral vectors. To increase the efficiency of transduction, we generated a CHO cell line that stably expresses ATRC-1, the receptor for ecotropic murine leukemia virus (Kim et al., 1991; Wang et al., 1991). The presence of ATRC-1 renders CHO cells highly permissive to retroviral transduction (data not shown). To test the efficiency of gene trapping, 50,000 CHO ATRC-1 cells were transduced with the ROSAβgeo * vector. Forty-eight hours after transduction, gene trap events were selected in the presence of G418. After 10 days of selection, cells were stained with Crystal violet in order to visualize the G418-resistant clones. A total of 30 G418-resistant clones were obtained. No clones were detected in control cells. It is estimated that a differentiated cell expresses an average of 10,000 genes at a given time. We reasoned that to cover the whole expressed genome, we should obtain about 50,000 to 100,000 gene trap events. We thus decided to scale up the experiment by a 3,000 factor, and transduced $1.5 \times 10^8$ cells, that in theory should give rise to approximately 90,000 gene trap clones. Immediately after transduction, cells were distributed in 122 24-well plates (i.e. 2928 wells) to ease further isolation of single clones (Fig. 2). Indeed, we reasoned that, given the efficiency of gene trapping and the likelihood of selecting an MHV68-resistant clone among them, it was unlikely to obtain more than one MHV68-resistant clone in a well. After 48 h, we started a concomitant selection for neomycin and MHV68 resistance. Two wells were selected with G418 only to verify that the gene-trapping efficiency was as expected. We performed a total of 3 rounds of infections with MHV68 over a 3-week period. Approximately 100 clones arose and were amplified. These clones were obtained in different wells and are thus independent, albeit possibly redundant, clones.

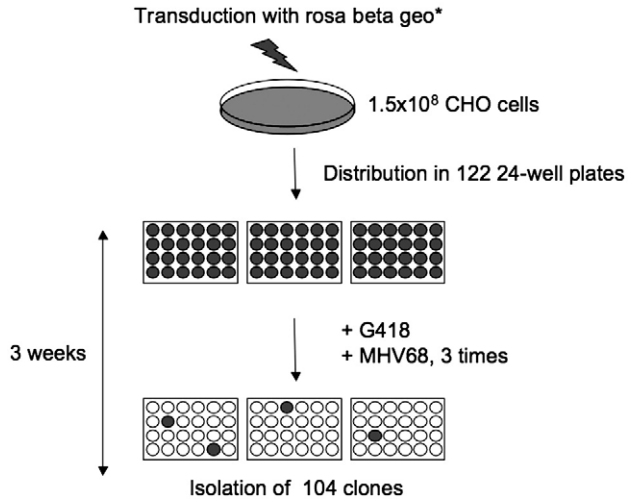


Fig. 2. Gene trapping mutagenesis and selection of MHV-68 resistant clones. CHO cells were transduced with the ROSAbetageo * gene trap vector and distributed in 24-well plates immediately after transduction, at approximately 50,000 cells per well. Forty-eight hours later, cells were selected in the presence of G418 and MHV68. Viral infections were repeated 2 more times over a 3-week period.

*Screening for clones that are affected in early viral events*

The clones obtained after our screening exhibited various degrees of resistance to MHV68-induced cytopathic effect when tested individually, the vast majority showing only a moderate resistance (i.e. cytopathic effect not as complete as wt cells). Two of the most resistant clones (i.e. total absence of cytopathic effect) are shown Fig. 3. We decided to focus our analysis on clones that were affected in the early steps of the viral cycle. For that purpose, we took advantage of the recombinant MHV68-GFP virus in which GFP expression is driven by a constitutive CMV promoter. After entry of the viral genome into the nucleus, GFP is expressed, independently of any MHV68 gene expression. Thus, GFP expression can be used as a marker to assess access of the viral genome to the nucleus. Each clone was infected individually with MHV68-GFP and the percentage of GFP-positive cells after 24 h was determined by flow cytometry. A sample of the analysis is shown Fig. 4. Most of the clones were not significantly different from the control wt CHO, indicating that these clones were able to support viral entry and access to the nucleus. By contrast, 13 clones were affected in early viral events as shown by either the absence or reduced number of GFP-positive cells (3 are shown Fig. 4: c1, c2 and c3). Unfortunately, our attempts to identify the gene defects in all these clones were unsuccessful. Characterization of the fusion transcripts by 5′RACE revealed the usage of a cryptic splice donor site present in the LTR region of the gene trap vector. This cryptic site was used instead of a cellular splice donor site and gave rise to a splicing between this region of the LTR and the splice acceptor site upstream of the selection

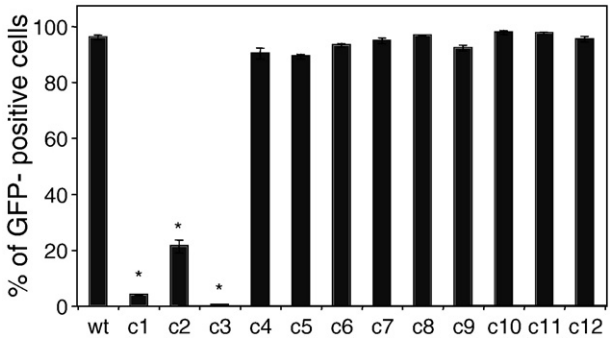


Fig. 4. Screening for clones showing defects in the initial steps of MHV68 infection. Wild type CHO and the gene trap clones were infected individually with MHV68-GFP at an MOI of 0.25. The percentage of GFP-positive cells was measured after 24 h by flow cytometry. Twelve clones are shown. * $p<0.001$ versus wt control.

gene (data not shown). This happens when an insertion occurs at the 5′end of genes (Medico et al., 2001), a situation that severely hampers the cloning of the trapped transcript by 5′ RACE.

*Role of the glycosaminoglycan heparan sulfate*

A likely possibility was that one, or several of these clones were deficient in cell surface expression of the glycosaminoglycan heparan sulfate (HS). Glycosaminoglycan chains on the cell surface proteoglycans provide initial docking sites for the binding to cells of various viruses and other microorganisms. In particular, numerous viruses (Liu and Thorp, 2002), including

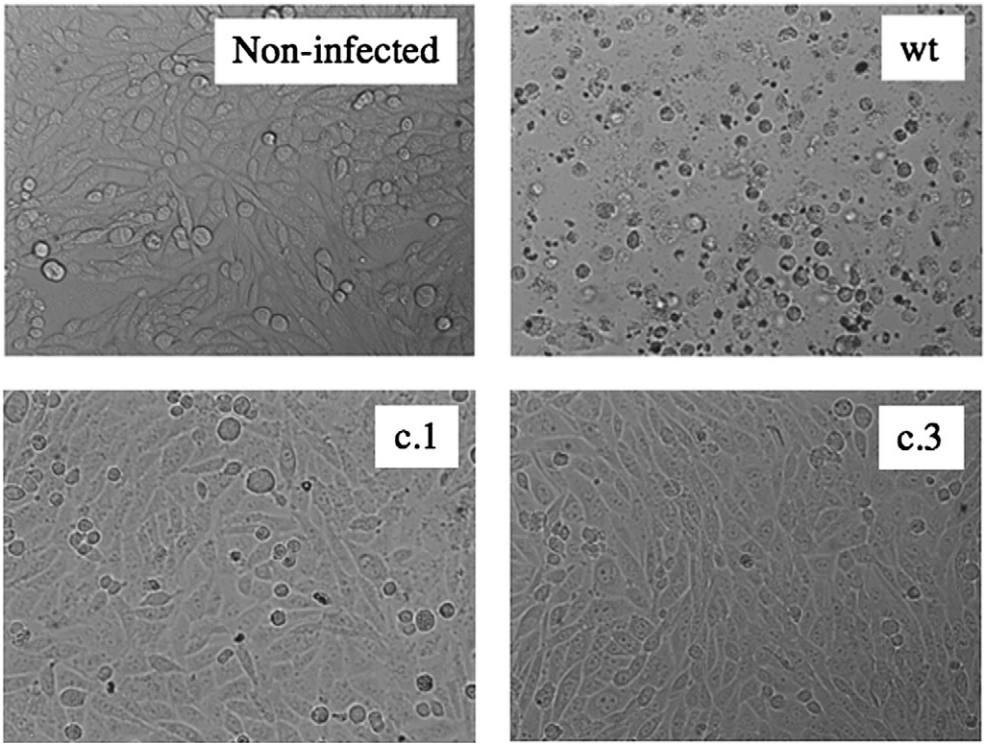


Fig. 3. Resistance to MHV68-induced cytopathic effect. Wild type CHO and the gene trap clones were infected individually with MHV68 at an MOI of 0.5. The presence (wt CHO) or absence (c1 and c3) of a cytopathic effect is shown by phase contrast microscopy. Two of the most resistant clones are shown.

herpesviruses (Shukla and Spear, 2001), have evolved to bind HS. Binding of viruses to HS provides a primary binding site that is thought to facilitate subsequent interaction with a more specific receptor that is necessary for viral entry. In most cases, deficiency in HS expression dramatically affects the efficiency of infection. To test if some of the CHO mutant clones were deficient in cell surface HS expression, we stained the cells with an antibody that recognizes HS and analyzed them by flow cytometry. We found that 10 clones were deficient for HS expression (2 of them are shown Fig. 5A). These corresponded to the ones that were completely resistant to the cytopathic effect. This finding confirms previous reports from Stevenson et al. showing that HS is a critical determinant for infection by MHV68 (de Lima et al., 2004; Gillet et al., 2007).

EXT1 is an enzyme involved in the biosynthesis of HS (Duncan et al., 2001; McCormick et al., 2000). Its absence leads to a total abrogation of HS synthesis (Lin et al., 2000). To test if any of the HS-deficient clones were affected in EXT1 expression, we transfected a vector encoding EXT1-GFP into these clones and tested for HS expression in the GFP-positive cells. Remarkably, we found that HS expression was restored in all the HS-deficient clones (Fig. 5B, only one clone is shown). By contrast, EXT2, a closely related enzyme that acts in cooperation with EXT1 (McCormick et al., 2000), had no effect on HS expression.

We performed an internalization assay to verify that viral entry was abolished in HS-deficient cells. Wild-type CHO or HS-deficient CHO cells (c3) were incubated with virus at 37 °C to allow internalization, or at 4°C as a negative control. After 90 min, surface-bound virus was removed by acid stripping and internalized viral genome was measured by quantitative PCR. No increase in the level of viral DNA was detected between 4 °C and 37 °C in HS-deficient cells (c3), whereas a significant increase was measured in wt cells (Fig. 5C). This result is consistent with the critical role of HS in viral entry.

To gain further insight into the mechanisms of viral binding at the cell surface, we decided to determine the nature of the heparan sulfate proteoglycan (HSPG) used by MHV68. Cell

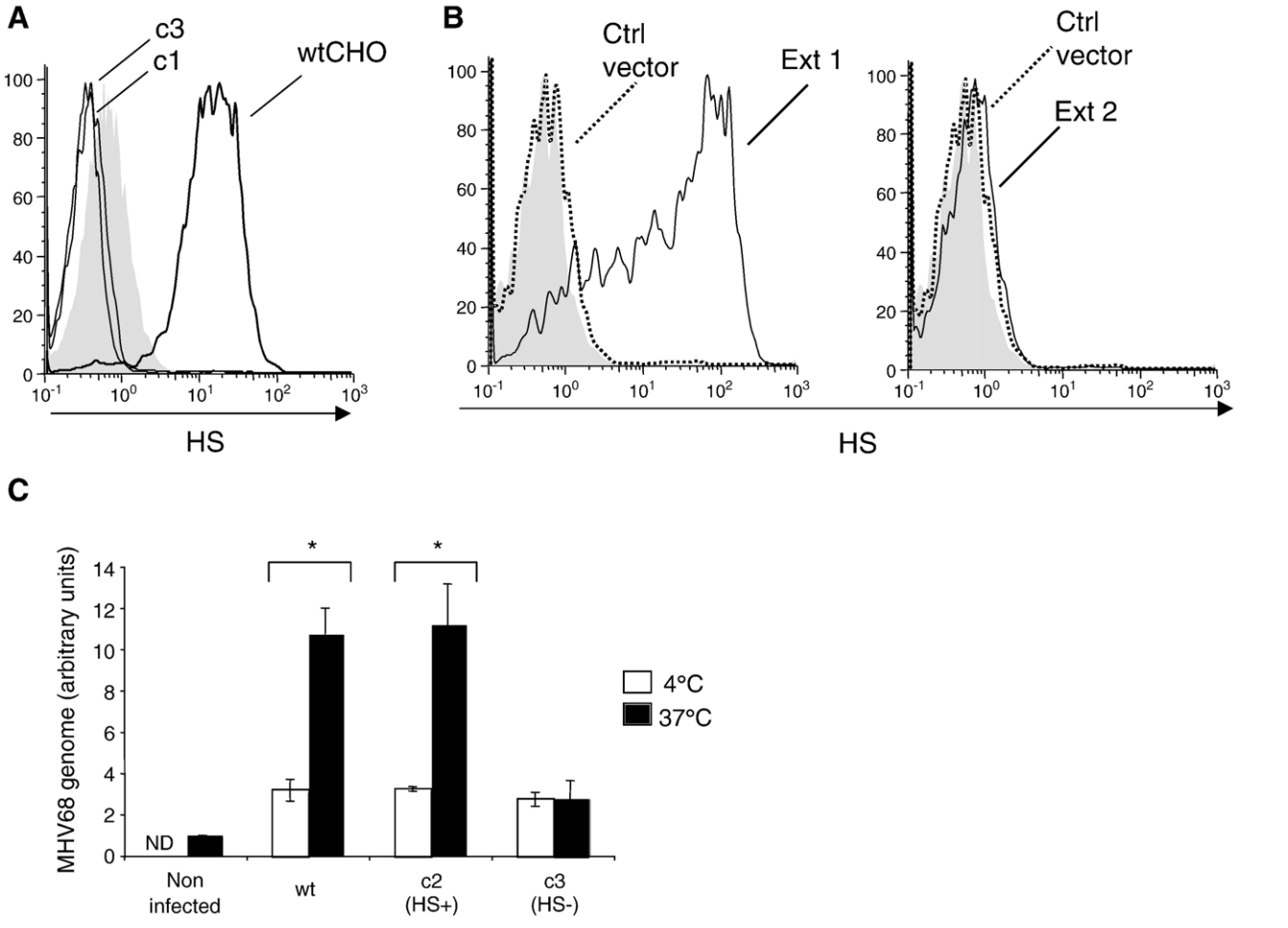


Fig. 5. Analysis of heparan-sulfate deficient clones. (A) WtCHO and the gene trap clones were tested for HS surface expression by flow cytometry. Two HS-deficient clones are shown (c1 and c3). The shaded histogram corresponds to a sample stained with an isotype control. (B) Heparan sulfate-deficient clones were transfected with an expression vector for EXT1-GFP, EXT2-GFP, or a control vector expressing GFP only. Twenty-four hours later, surface HS was determined by flow cytometry. The analysis is shown for one of the clones (c1), gated on the GFP-positive cells. The shaded histogram corresponds to staining with an isotype control. (C) MHV68 internalization was monitored in wtCHO, an HS-deficient clone (c3) and an HS-positive clone (c2), at either 4 °C or 37 °C. Internalized viral genome was quantified by real-time PCR. *$p<0.001$.

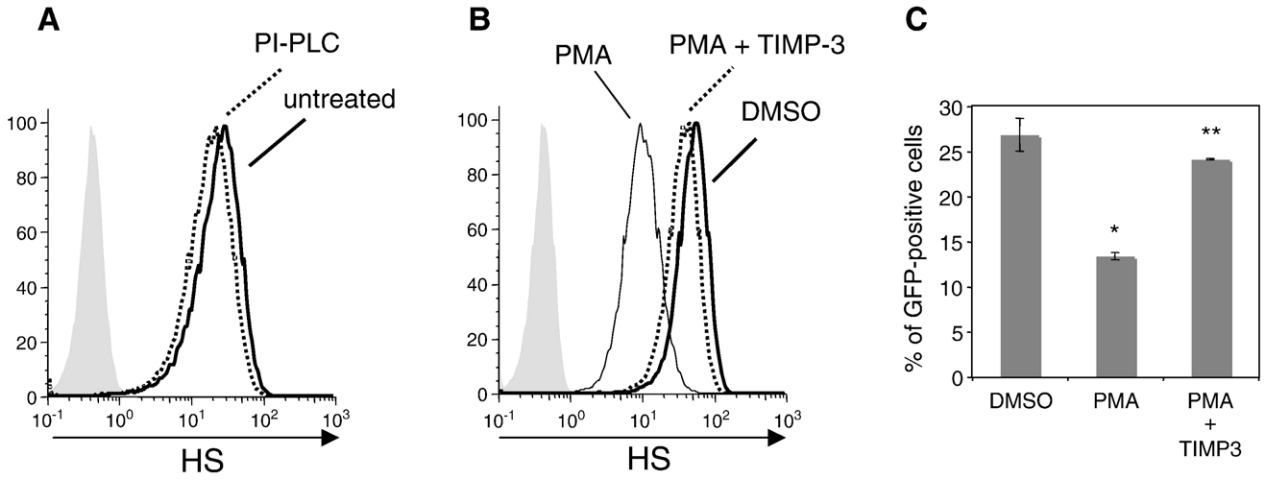


Fig. 6. Nature of the HSPG present on CHO cells. CHO cells were incubated for 1 h at 37 °C in the presence of 1 U/ml of PI-PLC (A), 1 μM of PMA (or same volume of DMSO as a control) or 1 μM PMA plus 10 μg/ml of TIMP-3 (B). After the drug treatments, HS surface expression was determined by flow cytometry. (C) Cells were treated as in panel B (DMSO, PMA, or PMA + TIMP-3). After 1 h, cells were infected with MHV68-GFP virus. GFP expression was determined by flow cytometry at 24 h post-infection. *$p<0.001$ versus DMSO control; ** not significantly different from DMSO control.

surface HSPG consist of heparan sulfate bound to primarily two families of proteins, the syndecans and the glypicans (Bernfield et al., 1999). The syndecans are a family of transmembrane proteins that are comprised of 4 members, whereas the glypicans are GPI-anchored proteins comprised of 6 family members. To test if HS was bound to glypicans on CHO cells, we treated the cells with a phosphatidylinositol-specific phospholipase C (PI-PLC) that cleaves GPI-anchored surface molecules. Enzyme efficacy was tested by removal of a known GPI-anchored protein (data not shown). As shown in Fig. 6A, PI-PLC did not significantly alter HS expression on CHO cells, indicating that the contribution of glypicans in surface HS was minor if any. Syndecans are constitutively shed into the extracellular medium (Kim et al., 1994; Spring et al., 1994), a process that can be accelerated by PMA (Subramanian et al., 1997) . Importantly, PMA-accelerated shedding of syndecans is specifically inhibited by the tissue inhibitor of metalloproteinase-3 (TIMP-3) (Fitzgerald et al., 2000). Thus, if HS is carried by syndecans, PMA treatment should result in a reduction in HS staining, in a TIMP-3 sensitive manner. Cells were incubated with PMA in the presence or absence of TIMP-3, and HS expression was assessed by flow cytometry. PMA treatment caused an 80% decrease in HS staining (Fig. 6B; mean fluorescence intensity: 48.5 for DMSO versus 9.64 for PMA), an effect that was abolished in the presence of TIMP-3. PMA-treated cells showed a significant reduction in their susceptibility to MHV68 (Fig. 6C). Full susceptibility was restored in cells that were incubated with PMA in the presence of TIMP-3. These results indicate that members of the syndecan family are a major source of HSPG used by MHV68 to infect CHO cells.

*Identification of early, post-entry defects*

Interestingly, c.2, c.15 and c.83 express normal level of HS (Fig. 7A), yet they are affected in the early stages of viral infection as shown by the reduced percentage of GFP-expressing cells following infection by MHV68-GFP (Fig. 7B). By contrast to the HS-deficient clones, c.2, c.15 and c.83 exhibited only a partial resistance, with c.2 and 15 showing the highest extent of resistance, and c.83 the lowest. Increasing the MOI increased susceptibility of c.2, c.15 and c.83 to levels comparable to that of wt CHO, by contrast to HS-deficient cells that remained resistant even at higher MOI (Fig. 7C). We further focused on c.2. Importantly, sub-cloning of this clone did not give rise to cells that were either totally susceptible or totally resistant, indicating that the partial resistance was not due to the presence of a mixed cell population (data not shown). We tested the efficiency of viral internalization in this clone and found that it was comparable to that found in wt CHO cells (Fig. 5C). These results indicate that c.2 is deficient for a factor involved in the early step of MHV68 infection, but downstream of viral internalization.

## Discussion

Here we report the characterization of CHO cell mutants that were isolated in a screen selecting for resistance to MHV68 infection. Although a high number of clones could be selected, the most resistant ones fell in the same category, i.e. clones deficient for HS expression. This strengthens the point that HS is a critical determinant of MHV68 infection. HS expression could be restored by transfection of EXT1, indicating that the lack of HS expression was likely due to a defect in EXT1 expression. Unexpectedly, this was true for all the HS-deficient clones obtained in our screen. This suggests the presence of a hot spot for either retroviral integration, or for the occurrence of spontaneous mutations, that alter expression of EXT1 (potentially at the *ext*1 locus or in genes controlling its expression). Although we did not identify the retroviral insertion site in these clones, we suspect that the high frequency of HS/EXT1 defects might be due to the occurrence of spontaneous mutations, as shown in L cells. Indeed, spontaneous L cell mutants defective for HS expression were isolated in an assay screening for

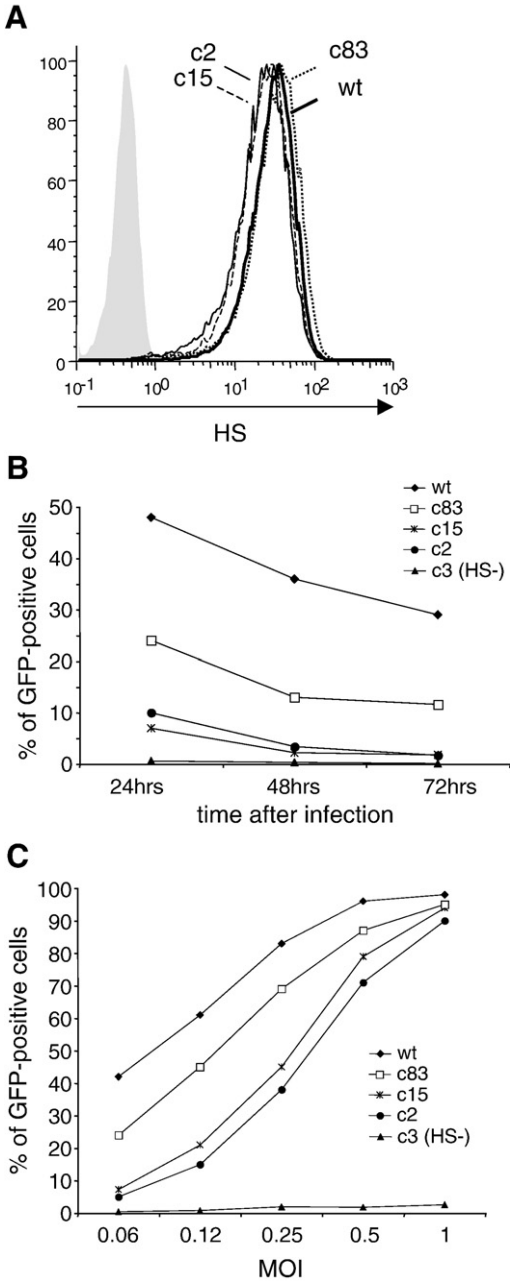


Fig. 7. Analysis of heparan sulfate-positive clones, affected at early stages of infection. (A) Three clones are shown that exhibit normal HS expression as monitored by flow cytometry. (B and C) WtCHO cells, an HS-deficient clone (c3), and the HS-positive clones c2, c15 and c83 were infected with MHV68-GFP and the percentage of GFP-positive cells was examined as before.

Herpes simplex virus-resistant cells (Banfield et al., 1995). HS expression could be restored by expression of EXT1 (McCormick et al., 1998). Together with the present study, these results argue in favor of spontaneous mutations naturally occurring in cell culture and that alter EXT1 expression. Unless there is a selection pressure, these mutants are not observed in normal growth conditions since the mutations do not confer a growth advantage. We believe that, with the development of promising new forward genetic systems, such as for example SILENCE (Banks and Bradley, 2007), the CHO system offers a powerful tool for genetic screens in mammalian cells. However, one must be aware that screens selecting for resistance to viruses, bacterias and even host-ligand binding, might be severely biased toward the selection of HS-deficient cells. Indeed, HS is required for the binding of many microorganisms but also of growth factors, chemokines and cytokines. Clonal selection, rather than bulk selection, should be used in order to prevent HS-deficient cells from taking over the culture in the detriment of partially resistant cells. We did a semi-clonal selection (90,000 gene trapped events distributed in approximately 3,000 wells before selection for MHV68 resistance). Among the 10 HS-deficient clones, 4 were heterogenous (i.e. a combination of normal HS-expressing cells and HS-deficient cells, data not shown). We believe this might be due to the appearance, during the 3 weeks of selection, of spontaneous HS-deficient cells in a population of partially resistant clones. This further confirms the strong predisposition toward the selection of HS-deficient cells. Finally, in light of our data, we believe that one way to overcome this setback might be to use CHO cells stably transfected with EXT1.

We confirm that heparan sulfate is a critical molecule required for infection by MHV68, consistent with previous reports from the Stevenson group (de Lima et al., 2004; Gillet et al., 2007) . Viral internalization was totally abolished in HS-deficient cells. The closely related human gammaherpesvirus, KSHV, is also critically dependent on HS for viral entry (Akula et al., 2001b). Heparan sulfate is used by many viruses, and microorganisms in general, to gain access to their target cells. The common view is that HS provides a docking site at the cell surface and by doing so, facilitates further interaction with a more specific receptor. However, the situation might be different in the case of MHV68. The envelope protein gp150 is the main determinant of HS dependence (de Lima et al., 2004). Yet, gp150 binds only weakly to HS, by contrast to gp70 that exhibits a strong affinity for HS (Gillet et al., 2007). Gillet et al. propose a model in which HS, rather than promoting adhesion per se, triggers a gp150 conformational change that activates viral entry. Premature engagement of gp150 would be prevented by gp70 binding to soluble HS. It would be interesting to see if this is a general control mechanism present in herpesviruses encoding several HS-binding proteins such as KSHV (Akula et al., 2001a; Birkmann et al., 2001; Mark et al., 2006; Wang et al., 2001).

Syndecans and glypicans are the two major families of HSPG at the cell surface (Bernfield et al., 1999). We provide evidence that MHV68 uses the syndecan family to infect CHO cells. Indeed, removal of the syndecans by PMA treatment reduced MHV68 infection, in a TIMP-3 sensitive manner. PMA treatment did not result in a complete removal of HS from the cell surface (20% left, Fig. 6B). Syndecans that were not shed during the time-lapse of PMA treatment could account for the remaining HS at the cell surface. Alternatively, a minor form of HSPG could contribute to HS expression. The TGF-β type III receptor, or betaglycan, has been shown to carry HS chains under certain conditions and is accordingly referred as a part-time HSPG (Iozzo, 2001), by contrast to syndecans and glypicans which are called full-time HSPG. Although HS has been thought to have a less significant role in ligand interactions

with betaglycan (Esparza-Lopez et al., 2001), we cannot totally exclude its potential contribution to viral infection of CHO cells, in addition to the syndecans. Finally, it should be noted that our study does not preclude the possibility that MHV68 could also use HS carried by glypicans in other cell types that express these HSPG.

In many studies reporting virus–HS interactions, the nature of the HSPG was not determined. However, in cases where the nature of the proteoglycan was examined (Jones et al., 2006; Kureishy et al., 2006; Saphire et al., 2001; Shafti-Keramat et al., 2003), syndecans were found to be used the most often. The structure of the core proteins might influence HS accessibility to viruses. The syndecans have an extended structure that is thought to position HS chains distant from the plasma membrane. By contrast, glypicans have a globular conformation with HS chains thought to lie closer to the cell surface. Alternatively, the fact that viruses seem to use preferentially HS carried by syndecans as coreceptors might be due to the ability of syndecans to mediate internalization of ligands (Williams and Fuki, 1997).

Clone 2 is affected in an early, post internalization event. The virus is internalized normally in this clone, but access to the nucleus is limited as shown by the reduced percentage of GFP-expressing cells upon infection with MHV68-GFP. The standard internalization assay that we used measures resistance of the viral genome to acid stripping. MHV68 has been shown to infect cells by endocytosis, with fusion events occurring in endosomes, at least in BHK21 cells (Gill et al., 2006). Virus trapped in endosomes would be acid-stripping resistant; thus one possibility is that c.2 has an intact machinery allowing internalization of MHV68 (i.e. access to an acid-stripping resistant compartment), but lacks a surface receptor necessary for viral fusion. Indeed, it is possible that interaction of MHV68 with HS present on syndecans triggers its endocytosis. The actual fusion events leading to the delivery of the nucleocapsid in the cytoplasm might involve interactions with a distinct receptor. Such receptor for MHV68 might be missing in c.2. Alternatively, transport of the nucleocapsid to the nucleus, or its interaction with the nuclear pore complex might be affected in c.2. Further characterization of this clone should give us insight into the molecular mechanisms involved in the early steps of gammaherpesvirus replication cycle.

## Materials and methods

### *Cells and viruses*

CHO-K1, NIH3T3 and BHK21 cells were from ATCC. The packaging Bosc cell line was a generous gift from Dr. William Sha (U.C. Berkeley, USA). CHO cells were maintained in RPMI 1640 medium with 10% (v/v) fetal bovine serum and penicillin–streptomycin. NIH3T3, BHK21, and Bosc cells were grown in DMEM supplemented as described above. MHV68-GFP was kindly provided by Dr. Herbert "Skip" Virgin (Washington University Medical School, USA). MHV68 and MHV68-GFP virus were produced by infection of BHK cells at a MOI of 0.05. After a complete cytopathic effect, the culture supernatant was titered using a standard plaque assay on NIH3T3 cells.

### *Plasmids, transfections and transductions*

The cDNA encoding for mouse ATRC-1, the receptor for ecotropic murine leukemia retrovirus, was a generous gift of Dr. Lorraine Albritton (University of Tennessee Health Science Center, USA). A *Bam*H1–*Eco*R1 restriction fragment encoding ATRC-1 was excised from pcDNA3 ATRC-1 and cloned into the retroviral vector PB-IP, a modified pBMN vector in which the gene for neomycin phosphotransferase gene has been replaced by the puromycin N-acetyl-transferase gene. The ROSAbeta geo * gene trap vector was kindly provided by Dr. Philippe Soriano (Fred Hutchinson Cancer Research Center, USA). pEXT1-GFP and pEXT2-GFP (McCormick et al., 2000) were kindly provided by Dr. Craig McCormick (Dalhousie University, Canada).

For transduction of PB-IP-ATRC-1, the packaging Bosc cell line (one well of a six-well plate) was transfected with 1 μg of retroviral vector mixed with 1 μg of a plasmid encoding the VSV-G protein (pMDG). Transfection was performed using the Fugene 6 reagent (Roche) according to the manufacturer's instructions. The virus-containing supernatant (2 ml) was harvested 48 h after transfection, filtered through a 0.45-μm filter and diluted with 8 μg/ml of Polybrene (Sigma). CHO cells (one well of a six-well plate) were transduced by spin infection (800×*g* for 2 h at 20 °C) and selection for transduced cells was started 48 h after transduction by addition of 10 μg/ml of puromycin.

For generation of the gene trap library, seven 15-cm dishes of Bosc cells were transfected as described above, using 105 μg of ROSAbetageo * mixed with 105 μg of pMDG. After 48 h, CHO cells from twenty 15-cm dishes were detached using PBS 2 mM EDTA ($1.5\times10^8$ cells), spun down, and the cell pellet was resuspended with the Bosc viral supernatant (210 ml) and polybrene. Cells were distributed in sixteen 6-well plates and centrifuged for 2 h as described above. Cells were then detached using trypsin and distributed in 122 24-well plates to achieve a density of approximately 50,000 cells per well. Forty-eight hours later, cells were selected in the presence of 500 μg/ml G418 and approximately 10,000 pfu of MHV68 per well. Viral infections were repeated after 1 and 2 weeks, using the same amount of virus (which corresponded to a much higher MOI due to the massive cell death induced by G418 and the initial MHV68 infection).

For transfection of EXT1-GFP, EXT2-GFP or a control empty vector (pIRES-GFP), HS-deficient CHO cells were plated in 6-well plates and transfected the next day using Fugene HD (Roche). Twenty-four hours later, cells were subjected to HS staining and flow cytometry analysis.

### *Flow cytometry analysis*

CHO cells were detached using calcium magnesium-free PBS 2mMEDTA. Cells were washed in PBS 1%BSA and incubated with an anti HS antibody used at 1:100 (10E4 epitope,

F58-10E4, from Seikagaku Corporation) for 30 min at 4 °C. The isotype control was a mouse IgMkappa (TEPC 183, from Sigma). After two washes in PBS 1%BSA, bound antibody was revealed by staining with a FITC- or PE -conjugated anti-mouse IgM antibody used at 1:300 (from eBioscience). Cell surface fluorescence was analyzed with a Becton Dickinson FACScalibur.

To examine viral infection using MHV68 GFP, CHO cells were plated in 12-well plates and infected 24 h later using 250 μl of virus diluted to achieve the indicated MOI. After 2 h at 37 °C, the viral inoculum was removed and replaced by 1 ml of fresh media. Before analysis of GFP expression by flow cytometry, cells were fixed in 4% paraformaldehyde.

*Internalization assay*

CHO cells were plated on 10 cm dishes ($10^6$ cells). The next day, cells were put on ice, rinsed with cold media and incubated with MHV68 at a MOI of 0.1, at 37 °C, or 4 °C as a negative control. After 90 min, cells were washed 4 times with cold PBS. Surface-bound virus was removed using two 5-min incubations with an acid-stripping solution (135 mM NaCl, 10 mM KCl, 40 mM Citric acid, pH 3). After 2 additional washes with PBS, cells were detached using trypsin and subjected to DNA extraction using Proteinase K (16 h at 50 °C) and phenol–chloroform extraction. MHV68 genome was quantified by real-time PCR using the SYBR Green system with an Applied Biosystems 5700 sequence detector. All samples were normalized to the signal generated from the house-keeping genes GAPDH or beta 2 microglobulin (both gave similar results). The following primers were used: MHV68 (mK3 gene), 5′-TGA TAC CCT TGG CTG TGC TGA TGA (forward) and 5′- AGA ACA CAA TCA TGG CCA GGA GGA (reverse); GAPDH, 5′-ACG TGT CCG TTG TGG ATC TGA CAT (forward) and 5′-AGC ATC AAA GGT GGA AGA GTG GGA (reverse); β2m, 5′-AGT CGA GCT GTC AGA TCT GTC CTT (forward) and 5′-TGG TGT GTG TAA CTC TGC AGG CAT (reverse).

*Drug treatments*

CHO cells were plated in 12 or 6-well plates. The next day, cells were rinsed with serum-free media and incubated with 1U/ml of PI-PLC (Sigma), 1μM of PMA (Sigma), or 1 μM of PMA plus 10 μg/ml TIMP-3 (recombinant human TIMP-3 from R&D systems) for 1 h at 37 °C. The control condition for PI-PLC treatment was serum-free media. We verified that 1U/ml of PI-PLC resulted in 100% removal of a known GPI-anchored protein (CD52 in mouse splenocytes, data not shown). The control for PMA was DMSO (same volume as PMA) in serum-free media.

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